NOVEL MUTATED MAMMALIAN CELLS
AND ANIMALS

The present application claims priority to U.S.
Provisional Serial Number 60/265,574 which was filed
January 31, 2001. The present application incorporates
U.S. Patent Nos. 6,080,576, 6,136,566, 6,139,833, and
6,207,371 and U.S. Applications Ser. Nos. 08/728,963,
60/109,302, and 09/276,533 and their respective
disclosures herein by reference in their entirety.

1.0. FIELD OF THE INVENTION

The present invention is in the field of molecular
genetics. The application discloses novel mutated cells
that are generated by a process involving the insertion
of at least a portion of a genetically engineered viral
vector into the chromosome. The specifically disclosed
recombinant vector allows for the rapid identification
of the gene that has been mutated by using nucleotide or
amino acid sequence information to identify the gene
that has been mutated by the vector. When mutated
embryonic stem cell clones are produced, such cells can
be used to produce mutant animals capable of germline
transmission of the described mutated genes.

2.0. BACKGROUND OF THE INVENTION

Most mammalian genes are divided into exons and
introns. Exons are the portions of the gene that are
spliced into mRNA and encode the protein product of a
gene. In genomic DNA, these coding exons are often
divided by noncoding intron sequences. Although RNA
polymerase transcribes both intron and exon sequences,
the intron sequences must be removed from the transcript
so that the resulting mRNA can be translated into
protein. Accordingly, all mammalian, and most

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eukaryotic, cells have the machinery to splice exons to produce mRNA. Gene trap vectors have been designed to insert into the introns of genes in a manner that allows the cellular splicing machinery to splice vector encoded exons to cellular mRNAs. Commonly, gene trap vectors contain selectable marker sequences that are preceded by strong splice acceptor sequences and are not preceded by a promoter. Thus, when such vectors integrate into a gene, the cellular splicing machinery splices exons from the trapped gene onto the 5' end of the selectable marker sequence. Typically, such selectable marker genes can only be expressed if the vector encoding the gene has integrated into an intron. The resulting gene trap events are subsequently identified by selecting for cells that can survive selective culture.

Gene trapping has generally proven to be an efficient method of mutating large numbers of genes. The insertion of the gene trap vector creates a mutation in the trapped gene, and also provides a molecular tag for ease of identifying the gene that has been trapped. When ROSAβgeo was used to trap genes it was demonstrated that at least 50% of the resulting mutations resulted in a phenotype when examined in mice. This indicates that the gene trap insertion vectors are useful mutagens. Although a powerful tool for mutating genes, the potential of the method has historically been limited by the difficulty in identifying the trapped genes. Methods that have been used to identify trap events rely on the fusion transcripts resulting from the splicing of exon sequences from the trapped gene to sequences encoded by the gene trap vector. Common gene identification protocols used to obtain sequences from these fusion transcripts include 5' RACE, cDNA cloning, and cloning of genomic DNA surrounding the site of

vector integration. However, these methods have proven labor intensive, not readily amenable to automation, and generally impractical for high-throughput.

More recently, vectors have been developed that rely on a new strategy of gene trapping that uses a vector that contains a selectable marker gene preceded by a promoter and followed by a splice donor sequence instead of a polyadenylation sequence. These vectors do not provide selection unless they integrate into a gene and subsequently trap downstream exons that provide a polyadenylation sequence. Integration of such vectors into the chromosome results in the splicing of the selectable marker gene to 3' exons of the trapped gene. These vectors provide a number of advantages. They can be used to trap genes regardless of whether the genes are normally expressed in the cell type in which the vector has integrated. In addition, cells harboring such vectors can be screened using automated (*e.g.*, 96-well plate format) gene identification assays such as 3' RACE (see generally, Frohman, 1994, PCR Methods and Applications, *4*:S40-S58). Using these vectors it is possible to produce large numbers of mutations and rapidly identify the mutated, or trapped, gene by DNA sequence analysis.

3.0. SUMMARY OF THE INVENTION

The subject invention provides numerous isolated mammalian mutant cell clones that are each characterized by the insertion of a mutagenic genetically engineered polynucleotide sequence into a gene identifiable as corresponding to one or more of the OMNIBANK gene trapped sequences (GTSs) disclosed in Sequence Listing.

The subject invention further contemplates a mutated cell, and particularly a mutated ES cell, and

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the animals derived from such ES cell that stably
maintain a genetically engineered mutation in a gene
identifiable as corresponding to one of the disclosed
GTSs.

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4.0. DESCRIPTION OF THE SEQUENCE LISTING AND FIGURES
     The Sequence Listing is a compilation of nucleotide
sequences obtained by sequencing clonal lines of gene
trapped murine ES cells.

10   Figures 1A-1C present a diagrammatic representation
of representative gene trap vectors used to generate the
described sequences.

     Figure 2 provides an index to the Sequence Listing
and the corresponding database accession numbers for the
15 genes that have been mutated according to the present
invention.

     5.0. DETAILED DESCRIPTION OF THE INVENTION
     The current invention relates to novel mutated
20 mammalian cells that are each characterized by the
insertion of a recombinant (i.e., genetically
engineered) mutagenic polynucleotide sequence into a
gene identifiable as corresponding to one of the GTSs of
SEQ ID NOS:1-248. For the purposes of the present
25 invention, the term "identifiable" is to be construed as
indicating that a mammalian cell, and preferably, a
murine ES cell, has been mutated by the insertion of a
polynucleotide sequence of recombinantly manipulated
origin at a genetic locus that normally comprises
30 polynucleotide sequence, and/or post-spliced exonic
sequence, that is at least partially described in one of
the GTSs of Sequence Listing. One method of determining
whether one of the described mutated mammalian cells has
a mutation in a gene of interest is by comparing the

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polynucleotide sequence (or a corresponding amino acid sequence) of the GTS identifying the mutated locus to the full length sequence of the gene. Alternatively, such searches can be conducted by comparing the described GTS sequence to a well known database (such as, but not limited to GENBANK) using established computer algorithms including, but not limited to, BLASTX, FASTA, BLASTN, BLASTP, TBLASTN, and TBLASTX using the default parameters used, for example, at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). The GTSs reported in the Sequence Listing have been compared to such a database (GENBANK), and the accession numbers of the genes that have been mutated are presented in Figure 2. Accordingly, an additional aspect of the subject invention includes mutated mammalian, preferably murine, cells, or isolated cell lines, that have at least one engineered mutation in a gene identified by a GENBANK or GENESEQ (for example) accession number in Figure 2.

As used herein, the terms "mutated" or "mutation" mean that the genetic locus has been altered by a process involving the integration or incorporation of a genetically engineered polynucleotide sequence into the genome of the cell with the result that the subsequent level of activity of the product normally encoded by the locus is altered (*i.e.*, reduced, increased, or substantially ablated). In those instances where the mutation substantially completely disrupts the expression or activity of the product normally encoded by the locus (*i.e.*, a null mutation), a cell that is heterozygous for the mutated allele will typically produce about one half of the product of a nonmutated cell (via a gene dosage effect).

The term "recombinantly manipulated" shall mean

that such compositions comprising such molecules or polynucleotides have been genetically engineered using molecular biology methodologies *in vitro* or *ex vivo* (see generally, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel *et al.*, 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.).

Where the specifically exemplified mammalian cells, *i.e.*, embryonic stem cells (Lex-1 cells from murine strain A129), are mutated by a process involving the insertion of at least a portion of a genetically engineered vector sequence into the gene of interest, the mutated embryonic stem cells can be microinjected into blastocysts that are subsequently introduced into pseudopregnant female hosts and carried to term using established methods such as those described in, for example, "Mouse Mutagenesis", 1998, Zambrowicz *et al.*, eds., Lexicon Press, The Woodlands, TX, and periodic updates thereof, herein incorporated by reference. The resulting chimeric animals are subsequently bred to produce offspring capable of germline transmission of an allele containing the engineered mutation in the gene of interest.

An alternative method of producing mutated cells and animals in the specifically exemplified genes involves the process of gene targeting by homologous recombination using methods such as those exemplified in U.S. Application Ser. No. 09/171,642, which is herein incorporated by reference in its entirety. Mutations produced using such methods include, but are not limited to, knockout mutations, "knockin" mutations (where a human gene, for example, is used to replace its murine ortholog), conditional, point mutations, and mutations

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that activate gene expression. Some of the mutations
described above (conditional mutations, point mutations,
etc.) can be produced via processes that involve the
substantial removal of vector encoded sequences (often
5   recombinase mediated) subsequent to the incorporation of
the recombinantly manipulated sequences into the genome.

The described mutated cells/animals can also be
used to predict drug action for compounds (small
molecules, antisense, antibodies, etc.) that inhibit the
10   activity or expression of the endogenous counterparts of
the described mutated genes/gene products.

5.1. MUTATED MAMMALIAN CELLS OF THE PRESENT
INVENTION The presently described mutated cells have
15   genetically engineered mutations in genes identifiable
as corresponding to, or normally comprising, at least a
portion of a sequence reported in the Sequence Listing
as SEQ ID NOS: 1-248. Additional embodiments of the
present invention are cells comprising engineered
20   mutations in homologs, paralogs, orthologs, *etc.*, of the
mutated genes disclosed in the Sequence Listing. Such
homologs, paralogs, and orthologs include genes having
sequences that hybridize to one or more of the disclosed
GTSs of SEQ ID NOS: 1-248 under stringent, or preferably
25   highly stringent, conditions. Hybridization conditions
also provide an alternative means of identifying the
mutated genes corresponding to the GTSs reported in the
Sequence Listing. Typically, such genes will be
identifiable because a disclosed GTS, or portion
30   thereof, will hybridize to the gene under stringent
conditions.

By way of example and not limitation, high
stringency hybridization conditions can be defined as
follows: Prehybridization of filters containing DNA to

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be screened is carried out for 8 h to overnight at 65°C
in a buffer containing 6X SSC, 50mM Tris-HCl (pH 7.5),
1mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500
μg/ml denatured salmon sperm DNA. Filters are

5   hybridized for 48 h at 65°C in prehybridization mixture
containing 100μg/ml denatured salmon sperm DNA and 5-20
x 10^6 cpm of ^{32}P-labeled probe (alternatively, as in all
hybridizations described herein, approximately 42, 44,
46, 48, 50, 52, 54, 56, 58, 62, 64, 66, 68, 70, or about

10   72 degrees or more can be used). The filters are then
washed in approximately 1X wash mix (10X wash mix
contains 3M NaCl, 0.6M Tris base, and 0.02M EDTA,
alternatively, as with all washes described herein, 2X,
3X, 4X, 5X, 6X wash mix, or more, can be used) twice for

15   5 minutes each at room temperature, then in 1X wash mix
containing 1% SDS at 60°C (alternatively, as in all
washes described herein, approximately 42, 44, 46, 48,
50, 52, 54, 56, 58, 62, 64, 66, 68, 70, or about 72
degrees or more can be used) for about 30 min, and

20   finally in 0.3X wash mix (alternatively, as in all final
washes described herein, approximately, 0.2X, 0.4X,
0.6X, 0.8X, 1X, or any concentration between about 2X
and about 6X can be used in conjunction with a suitable
wash temperature) containing 0.1% SDS at 60°C

25   (alternatively, approximately 42, 44, 46, 48, 50, 52,
54, 56, 58, 62, 64, 66, 68, 70, or about 72 degrees or
more can be used) for about 30 min. The filters are
then air dried and exposed to x-ray film for
autoradiography. In an alternative protocol, washing of

30   filters is done at 37°C for 1 h in a solution containing
2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is
followed by a wash in 0.1X SSC at 50°C for 45 min before
autoradiography. Another example of hybridization under
highly stringent conditions is hybridization to filter-

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bound DNA in 0.5 M NaHPO$_4$, 7% sodium dodecyl sulfate
(SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS
at 68°C (Ausubel F.M. *et al.*, eds., 1989, Current
Protocols in Molecular Biology, Vol. I, Green Publishing

5   Associates, Inc., and John Wiley & Sons, Inc., New York,
at p. 2.10.3).

Additional examples of GTS variants include
polynucleotides, or complements thereof, that are
capable of binding to the disclosed GTSs under less

10   stringent conditions, such as moderately stringent
conditions (*e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C
(Ausubel *et al.*, 1989, *supra*)). Moderately stringent
conditions can be additionally defined, for example, as
follows: Filters containing DNA are pretreated for 6 h

15   at 55°C in a solution containing 6X SSC, 5X Denhart's
solution, 0.5% SDS and 100 µg/ml denatured salmon sperm
DNA. Hybridizations are carried out in the same
solution and 5-20 x 10^6 cpm ^{32}P-labeled probe is used.
Filters are incubated in hybridization mixture for 18-20

20   h at 55°C (alternatively, as in all hybridizations
described herein, approximately 42, 44, 46, 48, 50, 52,
54, 56, 58, 62, 64, 66, 68, 70, or about 72 degrees or
more can be used in combination with a suitable
concentration of salt). The filters are then washed in

25   approximately 1X wash mix (10X wash mix contains 3M
NaCl, 0.6M Tris base, and 0.02M EDTA, alternatively, as
with all washes described herein, 2X, 3X, 4X, 5X, 6X
wash mix, or more, can be used) twice for 5 minutes each
at room temperature, then in 1X wash mix containing 1%

30   SDS at 60°C (alternatively, as in all washes described
herein, approximately, 42, 44, 46, 48, 50, 52, 54, 56,
58, 62, 64, 66, 68, 70, or about 72 degrees or more can
be used) for about 30 min, and finally in 0.3X wash mix
(alternatively, as in all final washes described herein

approximately 0.2X, 0.4X, 0.6X, 0.8X, 1X, or any
concentration between about 2X and about 6X can be used
in conjunction with a suitable wash temperature)
containing 0.1% SDS at 60°C (alternatively, approximately
42, 44, 45, 48, 50, 52, 54, 56, 58, 62, 64, 66, 68, 70,
or about 72 degrees or more can be used) for about 30
min. The filters are then air dried and exposed to x-
ray film for autoradiography.

In an alternative protocol, washing of filters is
done twice for 30 minutes at 60°C in a solution
containing 1X SSC and 0.1% SDS. Filters are blotted dry
and exposed for autoradiography.

Other conditions of moderate stringency that may be
used are well-known in the art. For example, washing of
filters can be done at 37°C for 1 h in a solution
containing 2X SSC, 0.1% SDS. Another example of
hybridization under moderately stringent conditions is
washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel *et al.*,
1989, *supra*). Such less stringent conditions may also
be, for example, low stringency hybridization
conditions. By way of example and not limitation,
procedures using such conditions of low stringency are
as follows (see also Shilo and Weinberg, 1981, Proc.
Natl. Acad. Sci. USA 78:6789-6792): Filters containing
DNA are pretreated for 6 h at 40°C in a solution
containing 35% formamide, 5X SSC, 50mM Tris-HCl (pH
7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500
μg/ml denatured salmon sperm DNA. Hybridizations are
carried out in the same solution with the following
modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA,
100μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate,
and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are
incubated in hybridization mixture for 18-20 h at 40°C
(alternatively, as in all hybridizations described

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herein, approximately 42, 44, 46, 48, 50, 52, 54, 56,
58, 62, 64, 66, 68, 70, or about 72 degrees or more can
be used). The filters are then washed in approximately
1X wash mix (10x wash mix contains 3M NaCl, 0.6M Tris
base, and 0.02M EDTA, alternatively, as with all washes
described herein, 2X, 3X, 4X, 5X, 6X wash mix, or more,
can be used) twice for five minutes each at room
temperature, then in 1X wash mix containing 1% SDS at
60°C (alternatively, as in all washes described herein,
approximately 42, 44, 46, 48, 50, 52, 54, 56, 58, 62,
64, 66, 68, 70, or about 72 degrees or more can be used)
for about 30 min, and finally in 0.3X wash mix
(alternatively, as in all final washes described herein,
approximately, 0.2X, 0.4X, 0.6X, 0.8X, 1X, or any
concentration between about 2X and about 6X can be used
in conjunction with a suitable wash temperature)
containing 0.1% SDS at 60°C (alternatively, approximately
42, 44, 46, 48, 50, 52, 54, 56, 58, 62, 64, 66, 68, 70,
or about 72 degrees or more can be used) for about 30
min. The filters are then air dried and exposed to x-
ray film for autoradiography. In yet another
alternative protocol, washing of filters is done for 1.5
h at 55°C in a solution containing 2X SSC, 25mM Tris-HCl
(pH 7.4), 5mM EDTA, and 0.1% SDS. The wash solution is
replaced with fresh solution and incubated an additional
1.5 h at 60°C. Filters are then blotted dry and exposed
for autoradiography. If necessary, filters are washed
for a third time at 65-68°C and reexposed to film. Other
conditions of low stringency that may be used are well
known in the art (e.g., as employed for cross-species
hybridizations). Preferably, GTS variants identified or
isolated using the above methods will also encode a
functionally equivalent gene product (i.e., protein,
polypeptide, or domain thereof, encoding or otherwise

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associated with a function or structure at least
partially encoded by the complementary GTS).

Low stringency conditions are well known to those
of skill in the art, and will vary predictably depending
on the specific organisms from which the library and the
labeled sequences are derived. For guidance regarding
such conditions see, for example, Sambrook *et al.*, 1989,
Molecular Cloning, A Laboratory Manual, Cold Springs
Harbor Press, N.Y.; and Ausubel *et al.*, 1989, Current
Protocols in Molecular Biology, Green Publishing
Associates and Wiley Interscience, N.Y.

The identification of homologs, heterologs, or
paralogs of SEQ ID NOS: 1-248 in other, preferably
related, species can be useful for developing additional
animal model systems that are closely related to humans
for purposes of drug discovery. Genes at other genetic
loci within the genome that encode proteins that have
extensive homology to one or more domains of the gene
products encoded by SEQ ID NOS: 1-248 can also be
identified via similar techniques. In the case of cDNA
libraries, such screening techniques can identify clones
derived from alternatively spliced transcripts in the
same or different species.

Techniques useful to disrupt a gene in a cell, and
especially an ES cell that may already have a disrupted
gene, are disclosed in U.S. Patent Nos. 6,136,566,
6,139,833 and 6,207,371, and copending US Patent
Application Ser. No. 08/728,963, each of which are
hereby incorporated herein by reference in their
entirety. Thus, it is also within the scope of the
current invention to disrupt a gene that encodes a
polynucleotide of the current invention.

5.2. USES OF THE DESCRIBED MUTATED GENES AND ANIMALS

The described mutated cells and animals are used to investigate and define the cellular and biological functions of the mutated genes. Producing a scientific model that accurately accounts for the large number of genes, proteins, and macromolecules within a single cell has thus far proved beyond the capabilities of existing computer technology. It should thus not be surprising that the far more complex task of modeling the various intricacies, cross and direct redundancies, and interrelated functions of the various metabolic and catabolic processes that occur within a single cell has also proven largely intractable to algorithmic methods of modeling and prediction. Even if one assumes that computer modeling of inherently chaotic/heuristic processes will rapidly mature in the near future, such methods, at best, can only provide predictions that subsequently require practical validation. Several decades of empirical data have proven that mutant phenotypes provide a valuable source of such validation.

The mutated diploid mammalian cells of the present invention will initially exist as mutated diploid cells that are heterozygous (except where genes on the X or Y chromosomes are mutated) for the mutations identified in the Sequence Listing. As such, via a "gene dosage" effect, the mutated cells can typically be characterized by the fact that they produce about one half of the mutated transcript/activity relative to cells having two nonmutated or wild type copies of the corresponding gene.

When mutant animals are produced from the mutated cells, heterozygous animals capable of germline transmission of the mutated allele can be bred to produce embryos or offspring that are homozygous for the

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mutant allele. Such animals or embryos are a rich source of tissues and cells that do not express physiologically relevant amounts of the mutated genes or activities encoded thereby. Accordingly, an additional embodiment of the present invention are mutant cells and animals that have homozygous mutations in genes identifiable as corresponding to the GENBANK, or other database accession, numbers provided in Figure 2, or are identifiable as a homologs, paralog, or orthologs of a sequence provided in the Sequence Listing.

In addition to providing important information regarding the functional role of a given gene in its nonmutated state (*i.e.*, learning about the function of the gene by discerning the effects of reducing or ablating the activity normally encoded by the gene), the described mutated cells and animals can be used as disease models, or in assays for compounds or genes (via gene delivery or transgenic methods) that compensate for the mutant phenotype and that can be used to treat diseases and disorders related to the observed phenotype. Alternatively, such products and genes can also be used to enhance desirable, if not normal, symptoms related to the observed phenotypes.

Another use for the presently described knockout cells and animals is the generation of high affinity antibodies against mammalian proteins. Given that the described knockout animals will never have seen the proteins expressed by the disrupted genes, the mutated animals can recognize mammalian orthologous proteins (including highly homologous human proteins) as foreign and mount an immune response against such proteins, whereas nonmutated animals might not. Such mammalian antibodies can be "humanized" using readily available means and used as therapeutic agents.

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The gene replacement/delivery therapies described above should be capable of delivering gene sequences to the cell types within patients that express the peptide or protein having the desired activity.

5   The examples below are provided to illustrate the subject invention. These examples are provided by way of illustration and are not included for the purpose of limiting the invention in any way whatsoever.

10   6.0.   EXAMPLES

6.1. GENERATION OF A LIBRARY OF MUTATED MOUSE ES CELLS
DEFINED BY GTS SEQUENCES

    VICTR retroviral vectors (e.g., VICTR 3, 20, BTK,
15 etc.), described in detail in U.S. Patent Nos.
6,080,576, 6,136,566, 6,139,833, and 6,207,371 were used
to generate a library of gene trapped ES cell clones
that represent a portion of the described GTSs. A
plasmid containing a VICTR cassette was constructed by
20 conventional cloning techniques and designed to employ
the features described above. Namely, the cassette
contained a *PGK* promoter directing transcription of an
exon that encodes an exon that ends in a canonical
splice donor sequence. Sequences were added to the
25 transcript containing the exon to allow for the
annealing of nested PCR and sequencing primers. The
vector backbone was based on pBluescript KS+ from
Stratagene Corporation.

    The plasmid construct was linearized by digestion
30 with *Sca*I, which cuts at a unique site in the plasmid
backbone. The plasmid was then transfected into the
mouse ES cell line AB2.2 by electroporation using a
BioRad Genepulser apparatus. After the cells were
allowed to recover, gene trap clones were selected by
35 adding puromycin to the medium at a final concentration

of 3 μg/ml (or other selectable agents such as, for
example, G418 were use as applicable depending on the
vector construct employed in the mutagenesis). Positive
clones were allowed to grow under selection for
5   approximately 10 days before being removed and cultured
separately for storage and to determine the sequence of
the disrupted gene.

Total RNA was isolated from an aliquot of cells
from each of 18 gene trap clones chosen for study. Five
10   micrograms of this RNA was used in a first strand cDNA
synthesis reaction using the "RS" primer. This primer
has unique sequences (for subsequent PCR) on its 5' end
and nine random nucleotides or nine T (thymidine)
residues on it's 3' end. Reaction products from the
15   first strand synthesis were added directly to a PCR with
outer primers specific for the engineered sequences of
puromycin and the "RS" primer. After amplification, an
aliquot of reaction products were subject to a second
round of amplification using primers internal, or
20   nested, relative to the first set of PCR primers. This
second amplification provided more reaction product for
sequencing and also provided increased specificity for
the specifically gene trapped DNA.

The products of the nested PCR were visualized by
25   agarose gel electrophoresis, and seventeen of the
eighteen clones provided at least one band that was
visible on the gel with ethidium bromide staining. Most
gave only a single band, which is an advantage in that a
single band is generally easier to sequence. The PCR
30   products were sequenced directly after excess PCR
primers and nucleotides were removed by filtration in a
spin column (Centricon-100, Amicon). DNA was added
directly to dye terminator sequencing reactions
(purchased from ABI) using the standard M13 forward

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primer, a region for which was built into the end of the *puro* exon in all of the PCR fragments.

Subsequent studies have used both VICTR 3 and VICTR 20. Like VICTR 3, VICTR 20 is exemplary of a family of vectors that incorporate two main functional units: a sequence acquisition component having a strong promoter element (phosphoglycerate kinase 1) active in ES cells that is fused to the puromycin resistance gene (or other exon sequence) that is followed by a synthetic consensus splice donor (SD) sequence and lacks an operatively positioned polyadenylation sequence downstream from the SD sequence (PGKpuroSD); and 2) a mutagenic component that incorporates a splice acceptor sequence fused to a selectable and/or colorimetric marker gene and followed by a polyadenylation sequence (for example, SAβgeopA, SAneopA, SAIRESneopA, or SAIRESβgeopA).

Also like VICTR 3, stop codons have been engineered into all three reading frames in the region between the 3' end of the selectable marker and the splice donor site. A diagrammatic description of the structure and functions of VICTRs 3 and 20 is provided in Figure 1.

When VICTRs 3, 20, and certain variations thereof, such as the vectors and methods described in U.S. Applications Ser. Nos. 09/276,533, and 60/095,989 (the disclosures of which are herein incorporated by reference in their entirety), were used in the commercial scale application of the presently disclosed invention, many mutagenized ES cell clones were rapidly engineered and obtained. Sequence analysis obtained from these clones has identified a wide variety of sequences. Each of the sequences presented in SEQ ID NOS: 1-248 identify novel mutations in the coding regions of mammalian genes that are identifiable as corresponding to the sequences presented in the Sequence

Listing. Alternatively, the described mutated cells are described by the database (GENBANK, GENSEQ, *etc.*) accession numbers for the corresponding genes that have been mutated (see Figure 2). The described mutated cells, and preferably ES cells, provide a valuable resource for defining, evaluating, or validating the biological function or disease/pharmaceutical relevance of each of these genes.

The cloned 3' RACE products resulting after the target ES cells were infected with one of the described gene trap vectors were purified using conventional column chromatography, (*e.g.*, S300 and G-50 columns), and the products were recovered by centrifugation. Purified PCR products were quantified by fluorescence using PicoGreen (Molecular Probes, Inc., Eugene Oregon) as per the manufacturer's instructions.

Dye terminator cycle sequencing reactions with AmpliTaq® FS DNA polymerase (Perkin Elmer Applied Biosystems, Foster City, CA) were carried out using approximately 7 pmoles of sequencing primer, and approximately 30–120 ng of 3' template. Unincorporated dye terminators were removed from the completed sequencing reactions using G-50 columns as described above. The reactions were dried under vacuum, resuspended in loading buffer, and electrophoresed through a 6% Long Ranger acrylamide gel (FMC BioProducts, Rockland, ME) on an ABI Prism® 377 with XL upgrade as per the manufacturer's instructions. The sequences of the resulting amplicons, or GTSs, are described in SEQ ID NOS: 1-248.

Suitable ES clones identified by the corresponding sequence tags can be microinjected into blastocysts, implanted into pseudopregnant females and used to generate chimeric animals that subsequently bred to

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product offspring capable of germline transmission of the mutated allele.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the above-described modes for carrying out the invention that are obvious to those skilled in the field of molecular biology or related fields are intended to be within the scope of the following claims.